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13. ABSTRACT (Maximum 200 Words)

The focus of this study is to gain insight into the role(s) of osteonectin in the preferential metastasis of breast cancer cells to bone. Osteonectin was isolated from conditioned media of several cell lines including breast cancer (MDA-MB-435, MDA-MB-468), osteoblasts (hFOB1.19), non-neoplastic breast epithelial (hTERT-HME1), and vascular endothelial cells isolated from bone biopsies (HBME-1). Analysis of translational and post-translational properties of osteonectin from these five sources revealed that a unique configuration of the protein does not exist; thus there is no detectable chemotactic isoform. Osteonectin increased motility of the breast cancer cells (MDA-MB-231) on culture plate surfaces. However, in transwell migration assays the MDA-MB-231 cells were not attracted to bone-derived osteonectin. Bone extracts from both wild-type and osteonectin-null mice had a profound stimulatory effect on migration in transwell chambers. We conclude that osteonectin does not stimulate breast cancer migration and therefore is not a chemotactic factor in the development of skeletal metastases. However, osteonectin enhances random undirected motility of breast cancer cells and therefore likely has a supportive role in breast cancer metastasis.

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Introduction

One in eight women will develop breast cancer in her lifetime [1] and many of these patients will suffer from bone metastasis [2]. Relatively little is understood about why bone is the preferred site of breast cancer metastasis. Osteonectin has been indicated as a chemoattractant for prostate cancer cells (which also preferentially metastasize to bone) [3] but whether osteonectin is a chemoattractant for breast cancer cells has not been thoroughly examined; this glycoprotein could be a major contributing factor to their preferential metastasis to bone. However, because bone cells and many metastatic breast cancer cells produce osteonectin, it is counterintuitive to postulate that breast cancer cells would be attracted to exogenous osteonectin. In order for a cell to migrate toward a chemotactic factor, a gradient must form so that a responsive cell can move toward greater concentrations. Because many metastatic breast cancer cells and bone cells produce osteonectin, a chemotactic gradient would not exist unless one of two conditions was present. The first condition is that the bone cells secrete a unique configuration of osteonectin, hence a chemotactic isoform. If tissue-dependent configurations of osteonectin exist, it is reasonable to hypothesize that bone-derived osteonectin could form a gradient and therefore attract breast cancer cells. The second condition is that the breast cancer cells that migrate to bone secrete little or no osteonectin and could thus respond to the bone-derived osteonectin. The purpose of this grant proposal was to characterize osteonectin from bone and breast cells to identify a chemotactic isoform. We also examined the ability of exogenous osteonectin to influence cell motility and migration of a breast cancer cell line that secretes low levels of osteonectin.

Body

Task 1: Study differences between breast cancer-derived and bone-derived osteonectin

The purpose of Task 1 was to identify a unique configuration of osteonectin. We accomplished this by analyzing post-translational modifications and translational differences of osteonectin from cell lines that represent both bone and breast cells. Specifically, we selected an array of cell lines that represent many of the cell types involved in breast cancer metastasis to bone. We used a non-neoplastic breast epithelial (hTERT-HME1) cell line and three human breast cancer cell lines (MDA-MB-435, MDA-MB-468 and MDA-MB-231); these cells provide a useful model of both normal and neoplastic breast tissue. To represent the bone microenvironment, we utilized a human bone marrow vascular endothelial cell line (HBME-1) and a human fetal osteoblast cell line (hFOB).

As stated in Task 1B, and reported in 2003, osteonectin was immunopurified from conditioned media collected from the MDA-MB-435, MDA-MB-468, hTERT-HME1, hFOB, and HBME-1 cell lines. Cells were grown to confluency, rinsed, and then exposed to DMEM/F-12 with Serum Replacement 3 (Sigma-Aldrich, St. Louis, MO) and 1% pen/strep for 24 hours. Osteonectin produced by the cultured cells was isolated using anti-human osteonectin mouse IgG (Haematologic Technologies, Essex Junction, VT) linked to an AminoLink® Plus Immobilization kit (Pierce, Rockford, IL). Approximately 250 ml of conditioned media was mixed with the antibody-coupled gel at 4°C overnight while rotating at 160rpm on a rotary platform. Samples were eluted from the gel with 0.1 M glycine, dialyzed against PBS, and stored at -80°C. Concentrations of osteonectin were determined by an ELISA (Haematologic Technologies).

Task 1A was accomplished by using immunopurified samples that were reduced with DTT, boiled, separated on a 15% SDS-PAGE gel and transferred to nitrocellulose membranes. The membranes were blocked with 3% bovine serum albumen (BSA) in phosphate buffered saline (PBS) for 1 hour at room temperature. Membranes were subsequently exposed to primary antibody (anti-human osteonectin mouse IgG, Haematologic Technologies) at a dilution of 1:15,000 in blocking solution and incubated overnight at room temperature. A secondary antibody, sheep anti-mouse IgG conjugated to horseradish peroxidase (HRP, Amersham Biosciences), was used at a dilution of 1:3750 in PBS and incubated for 1 hour at room temperature. The MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines all secrete detectable levels of osteonectin by immunoblotting with a molecular weight (~ 46 kD) (Figure 1). This data is contrary to the data reported in 2004 in which reduced osteonectin from these cell sources was described to have slightly different molecular weights. A 10% SDS-PAGE gel, used at that time was determined to be inadequate for good protein separation; in figure 1, we demonstrate better separation by using a 15% SDS-PAGE gel.

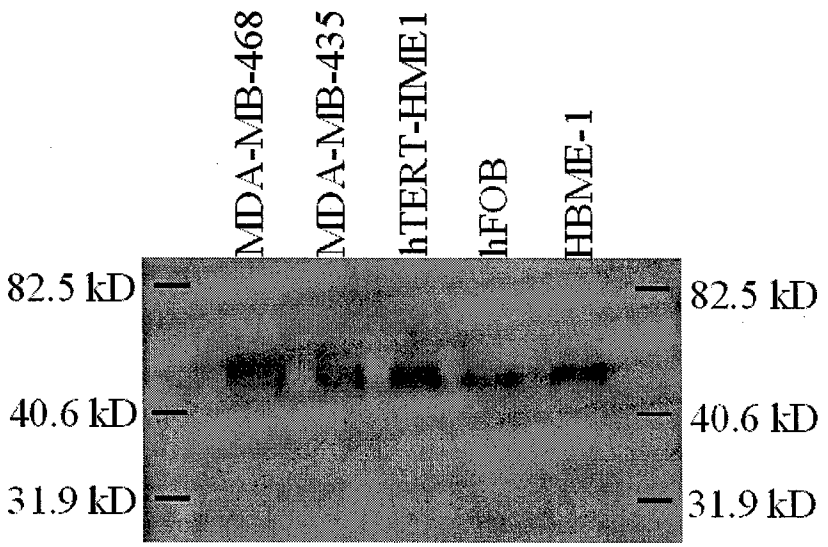

Figure 1 *Immunoblotting of osteonectin. Osteonectin from conditioned media was reduced and separated on a 15% SDS-PAGE gel. Osteonectin from the MDA-MB-468, MDA-MB-435, hTERT-HME1, hFOB and HBME-1 cell lines was detected at about 46kD.*

Although osteonectin from the various cell types has the same molecular weight, a chemotactic gradient could exist if one of the secreted forms has unique post-translational modifications. Therefore, we further analyzed osteonectin from these cell sources to identify specific post-translational modifications as specified in Task 1.

Two common post-translational modifications that could result in the formation of a unique configuration of osteonectin are glycosylation and phosphorylation. Task 1C is accomplished by the analysis of osteonectin glycosylation with an enzymatic protein deglycosylation kit (Sigma-Aldrich). This was reported in 2004 and replicated here. Briefly, 50 ng of sample was denatured and mixed with either a.) Reaction buffer alone (control), b.) PNGase, F c.) PNGase F with α-2 neuraminidase, d.) O-glycosidase, e.) O-glycosidase with α-2 neuraminidase, f.) O-glycosidase with α-2 neuraminidase and β-galactosidase, or g.) O-glycosidase with α-2 neuraminidase and β-N-acetylglucosaminidase. Samples were enzymatically deglycosylated at 37°C for 3 hours. The deglycosylated samples were then analyzed for shifts in migration by immunoblotting. In Figure 2, the glycosylation pattern of osteonectin secreted by the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines is presented. All of the sources of osteonectin exhibited a marked gel shift when exposed to PNGase F. This pattern of enzymatic deglycosylation demonstrates that the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines secrete osteonectin with N-linked oligosaccharides. There was no detectable gel shift to neuraminidase, O-glycosidase, β-galactosidase, or N-acetylglucosaminidase which remove sialic acid, O-link oligosaccharides, galactose, and β-linked N-acetylglucosamine residues, respectively. These data demonstrate that osteonectin from all cell lines examined have the same pattern of glycosylation.

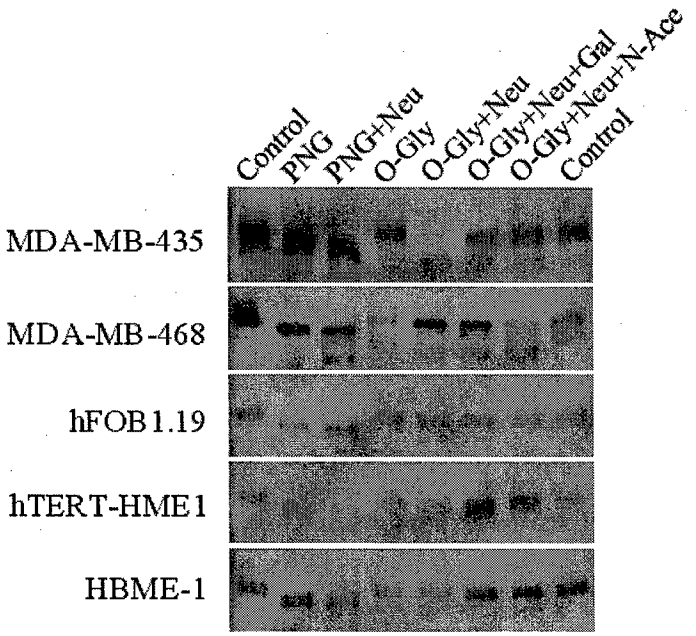

Figure 2 *Deglycosylation of osteonectin from different cell lines. Osteonectin was deglycosylated with a series of enzymes: PNGase F (PNG), neuraminidase (Neu), O-glycosidase (O-Gly), β-galactosidase (Gal), and N-acetylglucosaminidase (N-Ace). Samples were then separated on a 15% SDS-PAGE gel and immunoblotted. Compared to the control (no enzyme), the MDA-MB-435, MDA-MB-468, hFOB1.19, hTERT-HME1, and HBME-1 osteonectin displayed a molecular weight shift in response to PNGase F only.*

Task 1 is further accomplished by analysis of phosphorylation. Osteonectin has been described as a phosphoglycoprotein [4, 5] with multiple serine residues as possible phosphorylation sites [6]. We analyzed osteonectin from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines for the presence of phosphoserines. Detection of phosphoserines was accomplished by immunoblotting in the presence of phosphatase inhibitors (1 mM sodium orthovanadate and 10 mM sodium fluoride) in all the washes and antibody treatments. The primary antibody was an anti-phosphoserine mouse IgG (Sigma-Aldrich) diluted to 1:5000 in blocking solution. The secondary antibody was diluted to 1:3750 in PBS with 0.2% tween-20 and phosphatase inhibitors. Rat brain extracts (Biomol, Plymouth Meeting, PA) was used as the positive control. We determined that none of the tested osteonectin samples contain phosphorylated serines (Figure 3).

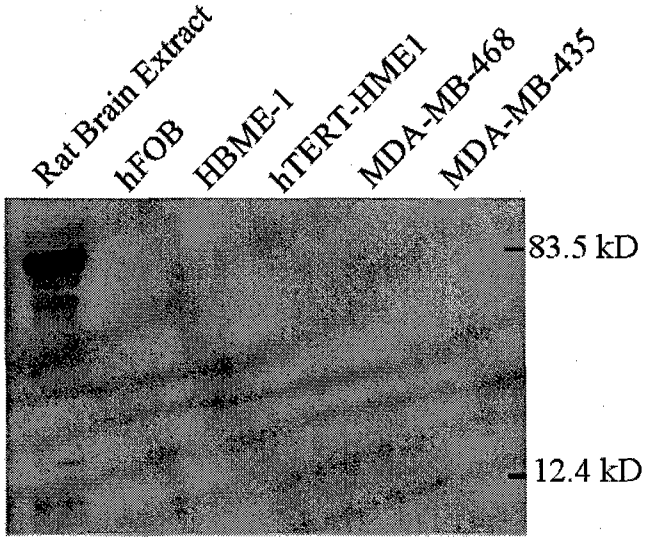

Figure 3 *Detection of phosphorlyated serines of osteonectin from different cell lines. Osteonectin from conditioned media was reduced and separated on a 12% SDS-PAGE gel. Protein loading was the same as in Figure 1. Osteonectin from the hFOB, HBME-1, hTERT-HME1, MDA-MB-468, and MDA-MB-435 cell lines was detected for phosphorylated serines. Rat brain extract was used as the positive control. None of the examined cell lines secrete osteonectin with phosphorylated serines.*

Because breast and bone cells secrete osteonectin with the same post-translational modifications, we then compared the core amino acid chain for translational differences. This was not originally part of Task 1 but fits into the scope; the purpose of Task 1 is to identify a unique configuration and we decided the best method to complete this task is to analyze the cDNA of osteonectin from the different cell lines. Briefly, cell cultures of MDA-MB-435, MDA-MB-468, hFOB, hTERT-HME1, and HBME-1 were rinsed in PBS and lysed; RNA was isolated and stored at -80°C. cDNA transcripts were generated by using the Ambion Retroscript kit (Austin, TX). The coding regions were amplified by RT-PCR using primers designed from the published human osteonectin sequence (Genbank accession # NM003118). PCR products were separated on a 1.5% agarose gel and visualized with ethidium bromide; bands were cut out of the gel and DNA was extracted. Both strands of the PCR products were then sequenced with internal primers that were designed approximately 400 base pairs apart to obtain overlapping sequence verification. The amino acid sequence was translated from the resulting cDNA sequence using the ExPASy web-based program (Figure 4).

We found the MDA-MB-435, MDA-MB-468, hFOB, and hTERT-HME1 samples to have identical cDNA sequences and therefore are assumed to have identical amino acid sequences to each other and to the published human osteonectin sequence (Genbank accession # NM003118). The HBME-1 cDNA sequence has a number of nucleic acid point mutations that resulted in eight amino acids that differed from the published sequence. The published osteonectin amino acid sequence with the eight substitutions

found in the HBME-1 sample is illustrated in Figure 4. The substitutions resulted in 1) threonine to alanine, 2) valine to glycine, 3) serine to proline, 4) aspartic acid to glutamic acid, 5) alanine to glycine, 6) glutamic acid to aspartic acid, 7) glutamine to glutamic acid, and 8) lysine to glutamine. However, at the amino acid level these differences appear minor. Substitutions # 1,3,4,5,6,7, and 8 are common evolutionary changes; these substitutions result in amino acids of similar polarity and mass and often do not affect the function of the protein [7]. There were also no changes in the number and position of cysteines which indicates that disulfide bonding was unaffected. There was a loss of a single serine (substitution 3) and a single threonine (substitution 1) which could affect phosphorylation. The EF-hands, which sequester Ca^{2+} and bind to other matrix proteins, were not affected by the mutations [8]. In addition, the GHK peptide within the follistatin-like domain that is responsible for stimulating angiogenesis and proliferation was also unaffected by the mutations [9].

We conclude that although the amino acid sequence of the HBME-1 derived osteonectin is different, the amino acid sequence of both breast cancer cells (MDA-MB-435 and MDA-MB-468), osteoblasts (hFOB), and non-neoplastic breast epithelial cells (hTERT-HME1) appear to be identical. Our final conclusion addressing Task 1 (to identify difference between breast and bone-derived osteonectin) is that these two cell sources secrete identical forms of osteonectin.

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    1           11          21          31          41          51
                                        1           2 3             4
1   MRAWIFFLLC  LAGRALAAPQ  QEALPDETEV  VEETVAEVTE  VSVGANPVQV  EVGEFDDGAE
                                                    5 6
61  ETEEEVVAEN  PCQNHHCKHG  KVCELDENNT  PMCVCQDPTS  CPAPIGEFEK  VCSNDNKTFD
                a
121 SSCHFFATKC  TLEGTKKGHK  LHLDYIGPCK  YIPPCLDSEL  TEFPLRMRDW  LKNVLVTLYE
                                                               b
181 RDEDNNLLTE  KQKLRVKKIH  ENEKRLEAGD  HPVELLARDF  EKNYNMYIFP  VHWQFGQLDQ
                                        c                   7 8
241 HPIDGYLSHT  ELAPLRAPLI  PMEHCTTRFF  ETCDLDNDKY  IALDEWAGCF  GIKQKDIDKD
301 LVI
```

Figure 4 *The amino acid sequence of published human osteonectin with the substitutions found in the HBME-1 osteonectin. MDA-MB-468, MDA-MB-435, hTERT-HME1, and hFOB osteonectin amino acid sequence is identical to the published sequence. There were eight substitutions identified in the amino acid translation of the HBME-1 sample, numbered 1-8. The substitutions are 1.) threonine to alanine, 2.) valine to glycine, 3.) serine to proline, 4.) aspartic acid to glutamic acid, 5.) alanine to glycine, 6.) glutamic acid to aspartic acid, 7.) glutamine to glutamic acid, and 8.) lysine to glutamine. The "GHK" (a), EF-hand 1 (b), and EF-hand 2 (c) peptides are highlighted.*

Task 2: Study the permeabilization of an endothelial layer due to the presence of breast cancer-derived osteonectin

For Task 2, we have been unable to demonstrate that osteonectin increases permeabilization of an endothelial layer. Interference reflection microscopy on an HBME-1 cell layer treated with 1μg/ml of MDA-MB-435-derived osteonectin failed to show any changes in cell shape, permeabilization or loss of focal adhesion complexes (Figure 5). Preliminary work on this concept was reported in 2004. We were unable to

show that breast cancer-derived osteonectin influences vascular endothelial cell permeability.

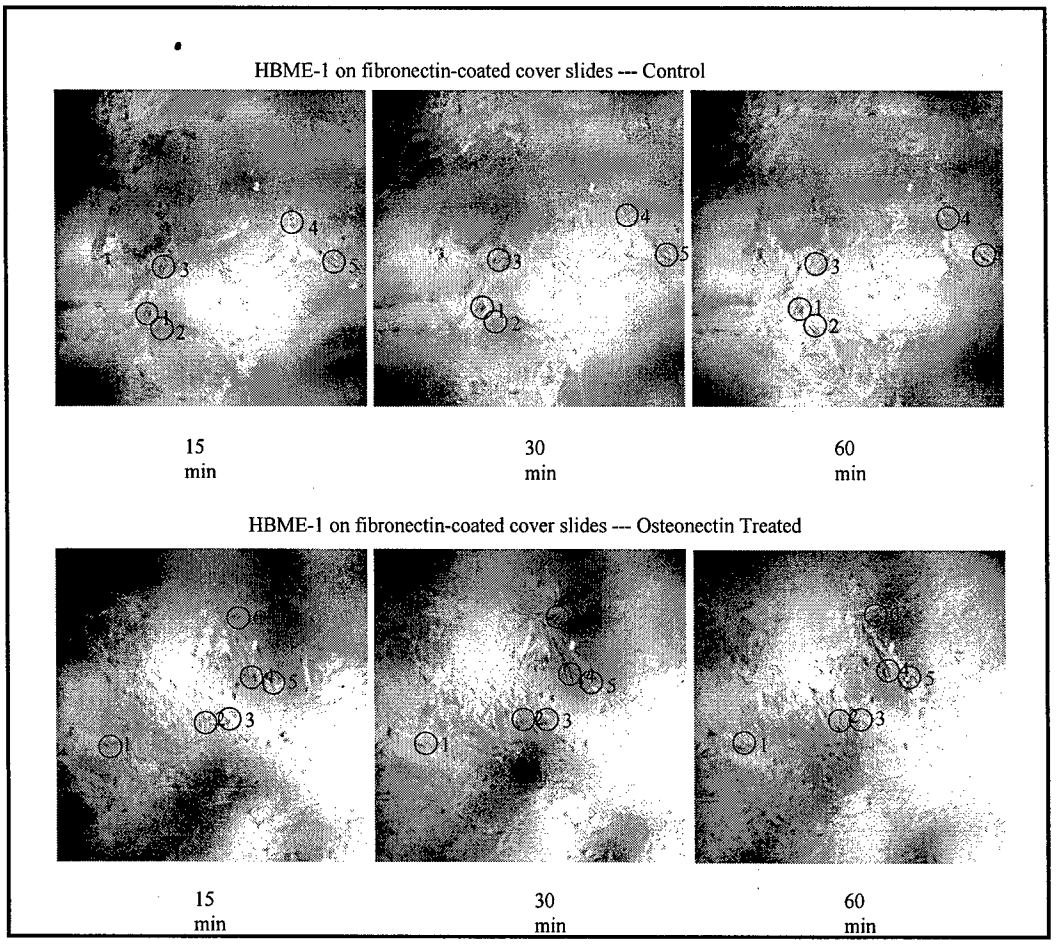

Figure 5 . Comparison of focal adhesion complexes in the presence and absence of osteonectin. After an hour with +/- 1μg/ml MDA-MB-435 osteonectin treatment, the number of focal adhesions in HBME-1 cells did not change. Glass cover slides are coated with 20μg/ml fibronectin (Biomedical Technologies Inc., Stoughton, MA). HBME-1 cells were grown to confluence, treated with either 0 or 1μg/ml MDA-MB-435 osteonectin.

Task 3: Study the chemotactic effect of bone osteonectin on breast cancer cells.

The purpose of Task 3 was to demonstrate the effects osteonectin has on cell motility and whether it directs breast cancer cell migration into bone through chemoattraction. Osteonectin has mainly been described as an anti-adhesive protein [10-12]; exogenous osteonectin can increase random undirected cell motility by inducing a state of intermediate adhesion. Intermediate adhesion is characterized by the ability of

cells to retain attachment to the matrix through integrins while focal adhesion plaques have disassembled. This type of adhesion is ideal for cell motility [11].

To test the ability of osteonectin to increase cell motility, we used the MDA-MB-231 cell line. This cell line secretes undetectable amounts of osteonectin and can therefore be used to study its response to osteonectin from another cell type. MDA-MB-231 cells were grown to confluency on 4-well permanox chamber slides (Nalge Nunc, International, Naperville, IL). A cross-shaped "wound" was created in each well by scraping the cell layer with a pipette tip (diameter ~ 0.6 mm). Detached cells were removed. The cross-shape wound gave a point of reference for two time points; images were collected at the same distance from the center of the cross-shaped wound at 0 and 6 hours in each chamber. DMEM/F-12 with serum replacement 3 and either vehicle (PBS only) or 500 ng of osteonectin from MDA-MB-468, hFOB or HBME-1 cells were added to each well. The chamber slides were incubated at 37°C in a 5% CO_2 humidified incubator. Wounds were viewed at 100X phase contrast magnification. Wound closure was calculated as percent change in the distance between the borders of cell growth. The MDA-MB-231 cells displayed greater cell outgrowth in the presence of osteonectin compared to the vehicle control (Figure 6). After 6 hours, there was no wound closure in the control treatment. However, the hFOB-derived osteonectin induced a 23% increase in wound closure and there was a 42% and 46% increase in wound closure by the HBME-1 and MDA-MB-468 derived osteonectin, respectively. These data support the literature that exogenous osteonectin enhances cell motility.

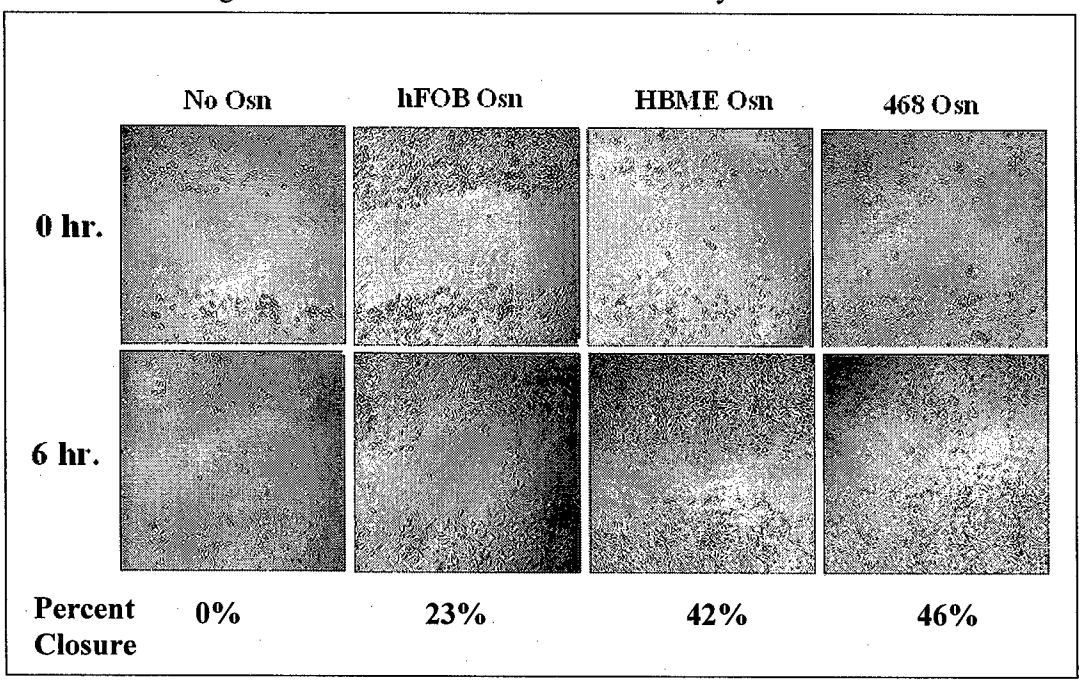

Figure 6 *Cell motility induced by osteonectin. MDA-MB-231 cells were grown to confluence on permanox chamber slides. A cross-shaped wound was then cut into the monolayer and the remaining attached cells were exposed to 500 ng of osteonectin from MDA-MB-468 (468), hFOB, HBME-1 (HBME) cells or control (PBS only) for 6 hours. The hFOB derived osteonectin induced a 23% wound closure while the HBME-1 and MDA-MB-468 osteonectin induced 42% and 46% closure, respectively. These images represent typical results from duplicate experiments.*

We then analyzed the ability of osteonectin to chemoattract breast cancer cells as stated in Task 3A. The migration of MDA-MB-231 cells, which do not secrete detectable levels of osteonectin, toward osteonectin isolated from the MDA-MB-435, MDA-MB-468, hFOB, HBME-1 and hTERT-HME1 cell lines was analyzed (Figure 7). The chemoattractant was diluted and air-dried to the lower surface of membranes in Falcon® FluoroBlock™ transwell chamber inserts (Becton Dickinson, Franklin Lakes, NJ). Osteonectin secreted from MDA-MB-435, MDA-MB-468, hFOB, hTERT-HME1 or HBME-1 was diluted in PBS; 0 ng (vehicle control), 25 ng or 50 ng of protein were air-dried to the lower surface of each well. Vibrant DiI stained MDA-MB-231 cells were seeded to the upper surface of the chamber at $5X10^4$ cells per well in the presence of 300 µl DMEM/F-12 with serum replacement 3 and 1% pen/strep. DMEM/F-12 (800 µl) was added to the lower chamber. After 6 hours, the membranes were then rinsed, fixed with 4% paraformeldahyde, and mounted for a microscopic examination. Migrated cells found on the lower surface of the membrane were visualized and counted with the use of 549 nm excitation and 565 nm emission wavelengths at 100X magnification. Each experiment was done in triplicate (total of n=9) and student t-test was used for statistical analysis. There was no significant migration toward either 25 ng or 50 ng of osteonectin from the MDA-MB-435, MDA-MB-468, and hFOB cell lines. There was some migration toward the highest concentration of hTERT-HME1-derived osteonectin (5-fold increase). However, the actual number of cells that migrate to the hTERT-HME1-derived osteonectin is very low, only about 10 cells migrated for every mm^2. There was also some migration to the HBME-1 derived osteonectin; we observed a 2-fold and 3-fold increase in migration toward the 25 ng and 50 ng concentrations, respectively. The number of migrated cells toward the HBME-1 derived osteonectin was also low. We observed about 4 cells in every mm^2 in the 25 ng concentration while the number of migrated cells toward the 50 ng concentration increased to about 6 cells per mm^2.

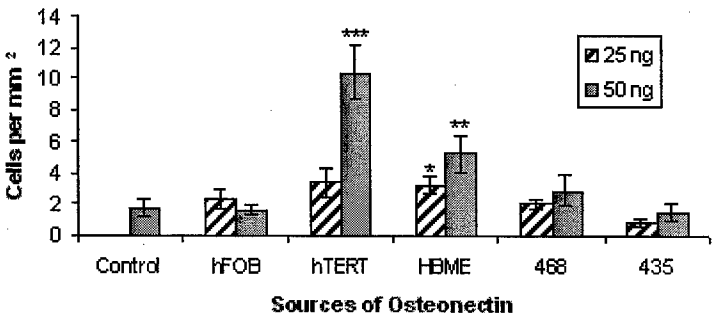

***Figure** 7 MDA-MB-231 cell migration toward osteonectin. Either 25 ng or 50 ng of osteonectin isolated from hFOB, hTERT-HME1, HBME, MDA-MB-468 (468), and MDA-MB-435 (435) cell lines was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells were added ($5X10^4$) to three membranes. After 6 hours, membranes were fixed and migrated cells were counted. Data represents three replicate experiments. There was significant migration to 50 ng of hTERT-HME1 osteonectin (5-fold increase). A 2-fold and 3-fold increase in migration was observed to 25 ng and 50 ng of HBME-1 derived osteonectin, respectively. No significant migration was observed toward the osteonectin isolated from the MDA-MB-435 (435), MDA-MB-468 (468), or hFOB cell lines. (N=9, mean ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$ compared to control)*

We further assessed the ability of bone-derived osteonectin to chemoattract breast cancer cells by using purified bovine bone-derived osteonectin (Hematologic Technologies). We extended the migration time to 48 hours and increased the concentrations of osteonectin to 50 ng, 100 ng, 200 ng, and 1 μg of protein per well. The metastatic breast cancer cells did not display any increase in migration to bone osteonectin (Figure 8).

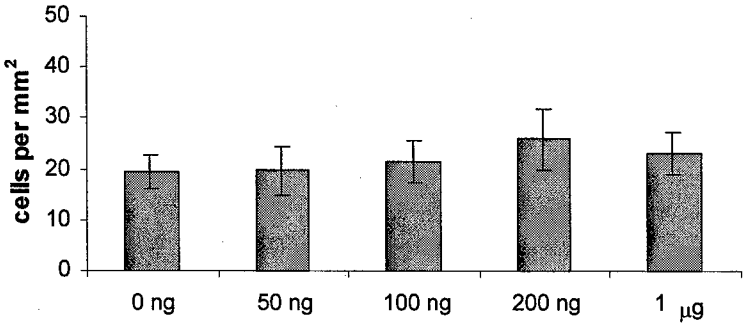

Figure 8 *MDA-MB-231 cell migration toward bovine bone osteonectin. Either 0, 50, 100, 200 ng or 1 μg of bovine bone was air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells were added ($5X10^4$) to three membranes. After 48 hours, membranes were fixed and migrated cells were counted. Data represents three replicate experiments. There was no significant migration to any concentrations of bone osteonectin (N=9, mean ± SEM)*

Physiologically, osteonectin exists in the presence of many other bone matrix proteins. The attractive quality of osteonectin may be dependent on its proximity to these other matrix proteins. As stated earlier, osteonectin increases cell motility only when the cells are bound to a matrix through integrins. The ability of osteonectin to act as a chemoattractant may rely on these same conditions.

We analyzed if osteonectin in the presence of other bone proteins could contribute to migration by utilizing bone extracts from wild-type and osteonectin-null mice. The femurs and tibias of 7-9 week old 129SV X C57BL/6 osteonectin knockout, heterozygous and wild-type mice were a gift from Dr. Hynda Kleinman (National Institutes of Health, National Institute of Dental and Craniofacial Research) [13]. Frozen bones were crushed into a fine powder using liquid nitrogen, mortar, and pestle. One gram of bone powder was diluted into 50 ml of extraction buffer (4.0 M guanidine HCl, 0.05 M Tris, 0.1 M 6-aminohexanic acid, 5 mM benzamidine HCl, and 1 mM phenylmethanesulfonyl fluoride (PMSF) pH 7.2) and mixed on a rotary platform at 160rpm for 24 hours in 4°C. Samples were centrifuged, the supernatant was removed, and a 10 ml volume of extraction buffer containing 0.5 M EDTA was added to the remaining residue. The samples were incubated at 4°C for 72 hours and mixed on a rotary platform at 160rpm. The samples were then centrifuged at 1800g and the supernatant collected. Bone extracts were dialyzed, lyophilized and reconstituted in dH_2O with protease inhibitors and stored at -80°C. We assayed the bone extracts for the

presence of osteonectin. In a SYPRO® stained SDS-PAGE gel, the wild-type sample has a single band that is noticeably absent in the knockout sample and reduced in the heterozygous sample. An immunoblot confirms the absence of osteonectin in the knockout sample (Figure 9).

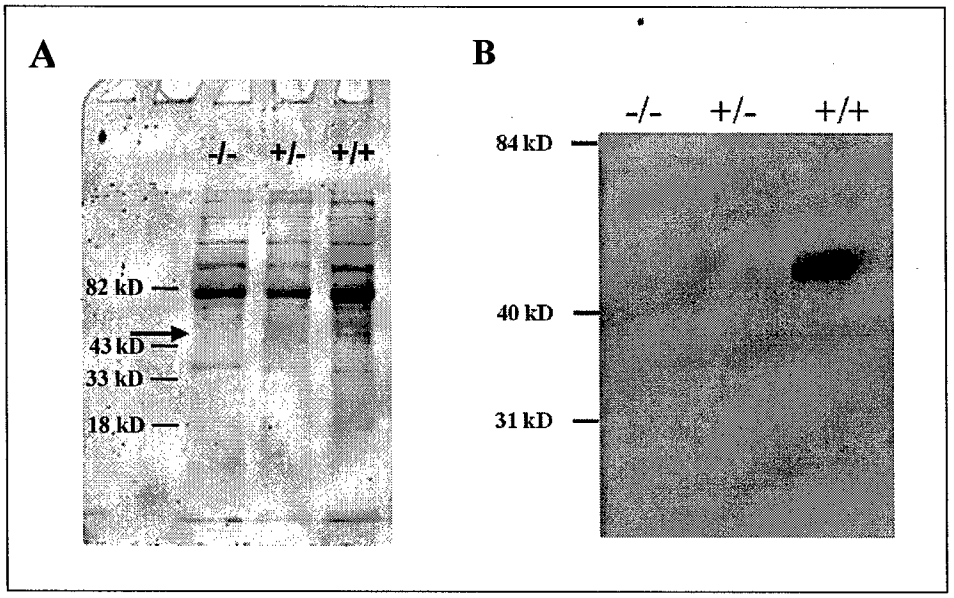

Figure 9 *Bone extracts from wild-type, heterozygous and osteonectin-null mice. A. The bone extracts were separated by SDS-PAGE and stained with SYPRO®. There was a single band absent (denoted with an arrow) in the knockout extract (-/-) and reduced in the heterozygous (+/-) sample. B. The bone extracts were analyzed by immunoblotting. Osteonectin was detected in the wild-type (+/+) and heterozygous extract but absent in the knockout sample.*

We then used the wild-type and osteonectin-null bone extracts in a transwell chamber migration assay. In Figure 10, the migration of MDA-MB-231 cells toward either vehicle (dH_2O with protease inhibitors) or 50 ng, 100 ng, and 200 ng of wild-type or osteonectin-null extracts was displayed. The cancer cells migrated to all concentrations of wild-type or osteonectin-null bone extracts. There was no difference in migration toward the wild-type or osteonectin-null extracts when compared at equal concentrations. This experiment indicates that the metastatic breast cancer cells are attracted to bone extracts independent of osteonectin. We conclude that in regards to Task 3, osteonectin increases random undirected cell motility but does not chemoattract breast cancer cells. Breast cancer cells are attracted to bone proteins but this occurs independent of osteonectin.

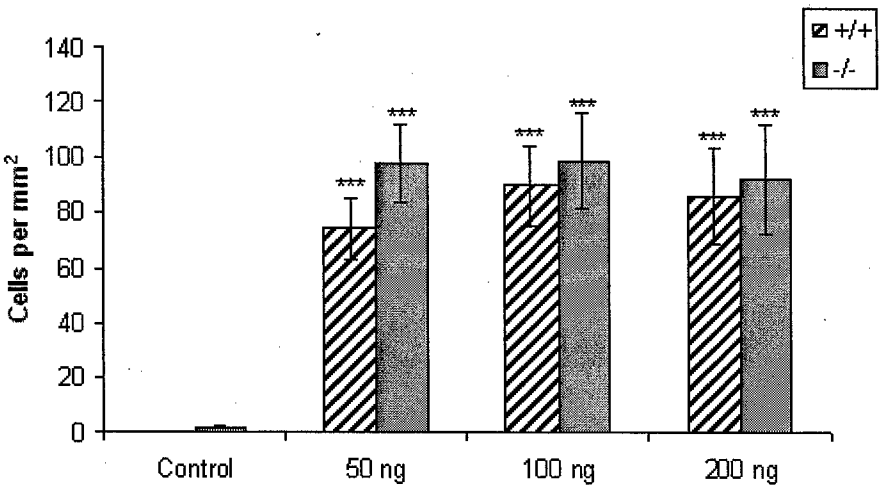

Figure 10 *Migration of MDA-MB-231 cells toward bone extracts. 0, 50, 100, or 200 ng bone extracts from the femurs and tibias of wild-type or osteonectin-null mice were air-dried to the lower surface of a transwell membrane. Vibrant® DiI stained MDA-MB-231 cells were seeded ($5X10^4$ cells per well) and allowed to migrate for 6 hours before being fixed. Data represents three replicate experiments. There was increased migration to all bone extracts (38-50 fold increase). No difference in migration toward the wild-type or osteonectin-null extracts of equal concentrations was detected. (N=9, mean ± SEM, *** $p \leq 0.001$ compared to control)*

Key Research Accomplishments

- Osteonectin from bone and breast cancer cells has a molecular weight of ~46kD.
- Osteonectin from bone and breast cancer cells have N-linked glycosylation and lack both sialic acids and O-linked oligosaccharides.
- Osteonectin from bone and breast cancer cells are secreted without phosphorylated serines.
- Bone and breast cancer cells produce identical osteonectin cDNA.
- Vascular endothelial cells from bone marrow produce osteonectin with a unique cDNA sequence.
- Bone-derived osteonectin is not a chemoattract for breast cancer cells.
- Non-neoplastic breast epithelial and vascular endothelial cell derived osteonectin may have some chemotactic quality.
- Breast cancer cells are attracted to bone extracts; but this occurs independent of osteonectin.

Reportable Outcomes

Manuscripts

Campo McKnight DA et al., An Evaluation of Osteonectin and its Role in the Attraction of Breast Cancer Cells to Bone. Submitted to J. Cell. Biochem.

Abstracts and Presentations

Campo DA, and CV Gay. Breast Cancer Cell Migration Is Stimulated By Bone-Derived Secretions Independent Of Osteonectin. Era of Hope, Department of Defense Meeting in Philadelphia, Pennsylvania, June 2005

Campo DA, DM Sosnoski, CV Gay. Comparison of bone-derived osteonectin and breast cancer secreted osteonectin; reducing and non-reducing conditions reveal distinct differences. Presented at the American Society for Bone and Mineral Research 26th Annual Meeting. Seattle, Washington. Published in J Bone Mineral Res. 19(Suppl 1): p227 2004.

Campo DA, DM Sosnoski, AM Mastro, DR Welch, CV Gay. Differences between osteoblast-secreted and breast cancer-secreted osteonectin; N-linked glycosylation may be key in chemoattraction. Presented at The Third North American Symposium on Skeletal Complications of Malignancy. Washington, DC. Published in Oncology, 17(Suppl 3): p20, 2003.

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NIH postdoctoral position in the Institute of Dental and Craniofacial Research in Bethesda, Maryland. Position begins in August of 2005 in the Craniofacial and Bone Diseases Branch.

Conclusion

We conclude that metastatic breast cancer cells (MDA-MB-435 and MDA-MB-468), osteoblasts (hFOB), non-neoplastic breast epithelial cells (hTERT-HME1) and vascular endothelial cells (HBME-1) secrete osteonectin that has similar or identical molecular weight (~ 46 kD). We also found that osteonectin from these cells has similar post-translational modifications. All forms of osteonectin tested in this study have similar glycosylation patterns, namely, all have N-linked oligosaccharides and have undetectable sialic acids and O-linked oligosaccharides. While the breast cancer, osteoblast and normal breast epithelial cell lines generated an identical osteonectin cDNA sequence, the vascular endothelial cells produce a distinctly different osteonectin cDNA; however, at the amino acid level these differences appear minor. Our investigations did not reveal notable differences between the osteonectin secreted by breast cancer cells or osteoblasts; therefore, a chemoattractant gradient based on a unique configuration of osteonectin is unlikely.

Osteonectin from osteoblasts, breast cancer cells and vascular endothelial cells increased MDA-MB-231 cell motility, as determined by the ability of osteonectin to enhance cell outgrowth in the wound healing assay. These results support the current literature that exogenous osteonectin enhances cell motility. However, the ability of a single concentration of soluble osteonectin to increase cell motility is distinctly different from chemoattraction, a process which is dependent on a gradient. To test for chemoattraction, or directed cell migration, cell movement toward a distant source of osteonectin needs to be tested.

Some migration of the MDA-MB-231 cells toward osteonectin from normal breast epithelial cells and vascular endothelial cells was observed, however, there was no migration to breast cancer or osteoblast-derived osteonectin in transwell chamber assays. The MDA-MB-231 cells did not respond to the bone-derived osteonectin even at a total protein level of 1 μg and a migration time of 48 hours. We conclude that pure bone-derived osteonectin does not attract breast cancer cells.

Despite the lack of migration to purified osteonectin, physiologically, osteonectin is in the presence of many other matrix proteins in the bone microenvironment. The attractive quality of osteonectin may be dependent on its proximity to these other matrix proteins. We analyzed if osteonectin in the presence of other bone proteins could contribute to migration by utilizing bone extracts from wild-type and osteonectin-null mice. We detected very high migration toward all bone extracts whether osteonectin was present or not. At equal protein concentrations, the metastatic breast cancer cells migrated to the wild-type or osteonectin-null bone extracts at the same rate. This experiment provides strong evidence that metastatic breast cancer cells are attracted to factors in the demineralized portion of the bone matrix. However, it also demonstrates that osteonectin is not a relevant contributor to the chemotaxis of breast cancer cells to bone.

This work is relevant to the scientific field to help clarify the effects osteonectin has on cancer cells. We established the difference between the ability of osteonectin to increase random cell motility rather than chemoattraction. Osteonectin does not direct cell motility and is most likely not a contributor toward breast cancer cell migration into bone. However, this study also shows strong evidence suggesting there are other factors with in the mineralized portion of the bone that do stimulate directed cell motility. Some

of these other factors within the mineralized matrix of bone could be bone sialoprotein and thrombospondin. Bone sialoprotein has been shown to increase MDA-MB-231 cell migration in an RGD-dependent manner [14]. Another study has demonstrated the chemoattraction of MDA-MB-435 cells toward thrombospondin-1 through the $\alpha_v\beta_1$ integrin [15]. Future work should include an in depth analysis into identifying which bone proteins are truly chemoattractive for breast cancer cells. A clear understanding of the factors directing breast cancer metastasis is the first step for the development of therapeutics and the prevention of metastasis.

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